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RESEARCH ARTICLE

RNA Polymerase II Dependent Crosstalk between H4K16 Deacetylation and H3K56 Acetylation Promotes Transcription of Constitutively Expressed Genes

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ABSTRACT Nucleosome dynamics in the coding region of a transcriptionally active locus is critical for understanding how RNA polymerase II progresses through the gene body. Histone acetylation and deacetylation critically influence nucleosome accessibility during DNA metabolic processes like transcription. Effect of such histone modifications is context and residue dependent. Rather than effect of individual histone residues, the network of modifications of several histone residues in combination generates a chromatin landscape that is conducive for transcription. Here we show that in *Saccharomyces cerevisiae*, crosstalk between deacetylation of the H4 N-terminal tail residue H4K16 and acetylation of the H3 core domain residue H3K56, promotes RNA polymerase II progression through the gene body. Results indicate that deacetylation of H4K16 precedes and in turn induces H3K56 acetylation. Effectively, recruitment of Rtt109, the HAT responsible for H3K56 acetylation is essentially dependent on H4K16 deacetylation. In Hos2 deletion strains, where H4K16 deacetylation is abolished, both H3K56 acetylation and RNA polymerase II recruitment gets significantly impaired. Notably, H4K16 deacetylation and H3K56 acetylation are found to be essentially dependent on active transcription. In summary, H4K16 deacetylation promotes H3K56 acetylation and the two modifications together work towards successful functioning of RNA polymerase II during active transcription.

KEYWORDS gene expression, H3K56, histone acetylation, H4K16, RNA polymerase II, transcription regulation

INTRODUCTION

For effective gene expression RNA polymerase II (RNAPII) is required to access DNA that is occluded by nucleosomes. In the promoter region of a locus while a stretch of nucleosome depleted region (NDR) is generated for RNAPII access, the coding region has localized disruption of nucleosomes that is enough for RNAPII to progress.[1–6] The accessibility of nucleosomal DNA is critically regulated by post-translational modification of histones.[7,8] Of the known post-translational histone modifications, acetylation of lysine residues is the most prevalent.[9] Some of the prominent acetylation events involve residues of the histone H4 N-terminal tails, including that of H4K16. Being the only acetylable residue present in the acidic patch binding region of histone H4 tail, H4K16 acetylation can directly influence higher order chromatin compaction-relaxation dynamics.[10–13] The state of H4K16 acetylation is critically regulated and affects gene silencing, transcription, as well as DNA repair.[14–19] Besides affecting local chromatin dynamics,[20] histone tail modifications primarily function to recruit chromatin effector proteins that in turn alter nucleosome structure and affect different stages of transcription.[21–24] At the nucleosomal level, often more than the histone tail residues those

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present on the globular core domain can directly influence interactions between the histone octamer and the nucleosomal DNA.[25–27] H3K56 is a globular core residue located at the site where the nucleosomal DNA enters or leaves the histone octamer and its acetylation weakens the interaction at the DNA entry/exit site thereby promoting unwrapping of nucleosomal DNA.[28–32] Thus far, acetylation of H3K56 is known to primarily occur on free histones, in a replication-dependent or independent manner, and is accomplished in yeast by a complex formed by the histone chaperone Asf1 and the HAT Rtt109.[30,33–37] In *Saccharomyces cerevisiae*, H3K56 acetylation is a marker of active transcription that has been strongly associated with promoter-nucleosome dynamics, chromatin disassembly and histone exchange.[35,38–41] By exposing the linker DNA region, H3K56 acetylation is known to maintain chromatin in a more poised state and facilitates recruitment of the SWI/SNF chromatin remodeling complexes.[42–44] Furthermore, in heterochromatin loci, H3K56 acetylation can promote transcription elongation without affecting the formation of higher order chromatin structures.[45] In ddition, histone H2A/H2B eviction and FACT mediated nucleosome assembly/disassembly is critically regulated by H3K56 acetylation.[46,47] Recent in vitro studies have suggested that by unwrapping nucleosomal DNA H3K56 acetylation can shorten the pause duration of RNAPII.[48] Indeed, in mESCs, it has been reported that hyperacetylation of H3K56 facilitates promoter-proximal pause release and RNAPII progression within genes.[49] Thus, acetylation of histone tail and core domain residues confers distinct modes of chromatin regulation that alters nucleosomal DNA accessibility and affects processes like transcription. Often histone modifications do not act in isolation, but their deposition, recognition and influence on chromatin functions are dependent on the crosstalk with modifications on other histone residues.[50,51] For instance, Set 1 mediated H3K4 trimethylation which is a hallmark of active transcription is dependent on H2B ubiquitination.[52,53] Conversely, H3K9 acetylation is dependent on H3K4 trimethylation.[54,55] In mammals for instance, H3K27 acetylation is required for H3K4 trimethylation, and H3K4 trimethylation alone cannot promote gene activation in absence of H3K27ac.[56] Although histone acetylation is often associated with active genes in yeast, role of H4K16 deacetylation has been found to be unique. Interestingly, presence of H4K16 deacetylation with H3K36me3 signals transcription elongation, while H4K16 deacetylation with H3K4me3 suppresses transcription initiation.[57] Thus, a particular histone modification may signal both activating and repressive functions based on its crosstalk with associated histone modifications. Often these histone modification cross talks are cotranscriptionally established on chromatin based on the RNAPII CTD phosphorylation status [53,58–65] and serves to orchestrate an intricate array of signals that regulate distinct stages of transcription. Thus, understanding how RNAPII mediates the crosstalk between histone post-translational modifications serves as the basis for deciphering the exact histone code that effectively regulates transcription.

In this study, we elucidate a functional crosstalk between deacetylation of H4K16 and acetylation of H3K56 during transcription of constitutively expressed genes. We demonstrate that in *S. cerevisiae*, this cross talk is essentially dependent on active RNAPII with H4K16 deacetylation being an important prerequisite for H3K56 acetylation. Our work indicates that the RNAPII mediated crosstalk between H4K16 deacetylation and H3K56 acetylation is essential for efficient transcription in the gene body of constitutively expressed genes.

RESULTS

Hyperacetylation of H3K56 in the ORF region promotes expression of constitutively active genes. To understand the significance of H3K56 acetylation in regulating expression of constitutively active genes, we performed ChIP assay with antibody against acetylated H3K56. We observed that in the ORF of constitutively active genes H3K56 gets hyperacetylated, relative to the promoter region (Fig. 1A). To endorse that the observed H3K56 acetylation was actually related to active transcription, H3K56 acetylation levels in the transcriptionally silenced telomere locus (*TEL08R*),

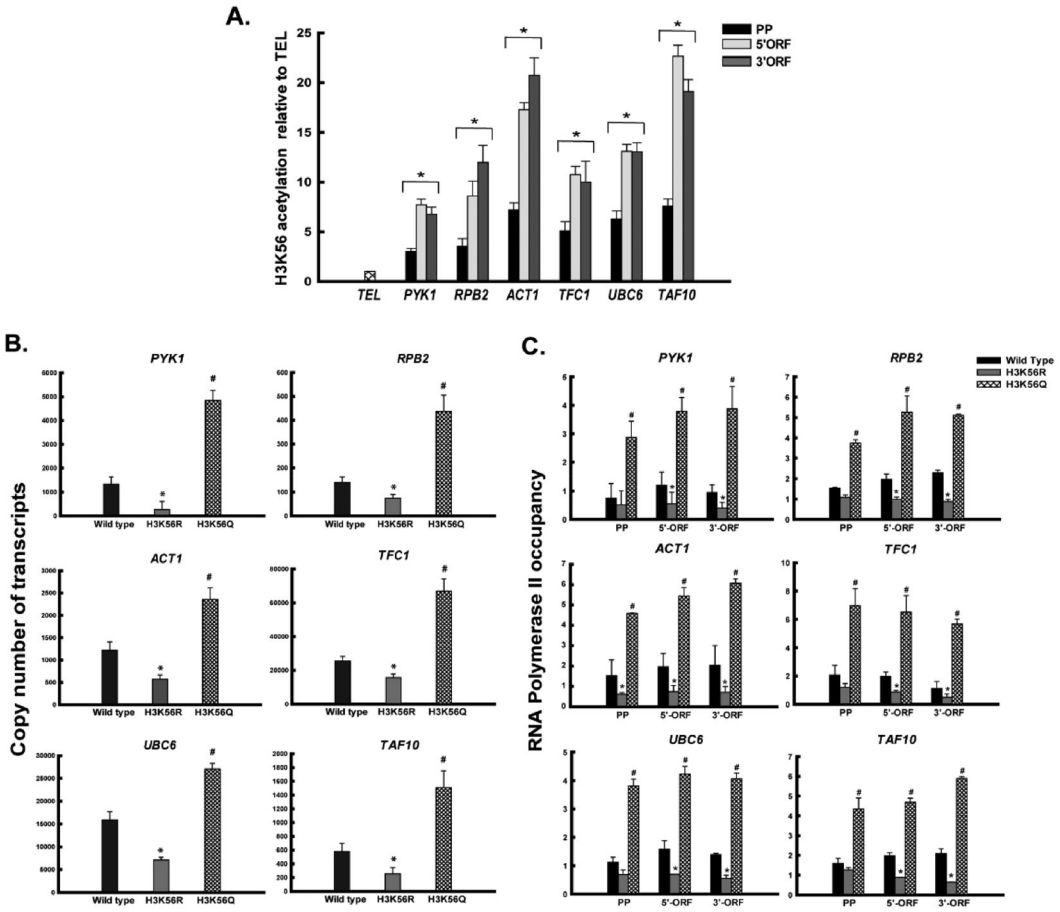

FIG 1 H3K56 acetylation is required for transcription of constitutively expressed genes. (A) ChIP qPCR analyses were performed to check H3K56 acetylation levels in the promoter (PP), 5′ORF and 3′ORF of *PYK1, RPB2, ACT1, TFC1, UBC6* and *TAF10* loci. H3K56 acetylation levels were normalized to ChIP data with anti-H3 antibody of the same regions and graphically represented relative to a nontranscribing control region of *TELO8R* denoted as *TEL*. (B) RNA was isolated from wild-type and H3K56 mutants followed by RT-qPCR analyses using absolute quantification method as described in Materials and Methods. The copy number of transcripts in wild-type and mutants, respectively are graphically plotted for the genes mentioned above. (C) ChIP analyses with anti-RNAPII (8WG16) antibody to check occupancy at the promoter (PP), 5′ORF and 3′ORF of *PYK1, RPB2, ACT1, TFC1, UBC6* and *TAF10* loci. The data normalized by 1% input was graphically plotted. Data represents the mean of three independent experiments with standard error of mean bars. * and # denote *t* test significant *P* values of <0.05.

was used as the control. To further correlate the role of H3K56 acetylation with transcription of active genes, we worked with two mutants – H3K56R, which mimics a state of constitutive deacetylation and H3K56Q, mimicking a state of constitutive acetylation. We performed RT-qPCR based comparative gene expression analyses between wild type and the two mutant strains of H3K56. Our results indicated that absence of H3K56 acetylation (H3K56R) caused significant reduction in gene expression levels, compared to wild-type. On the contrary, presence of constitutive acetylation of H3K56 (H3K56Q) resulted in significant upregulation of gene expression, relative to wild-type (Fig. 1B). It was further observed that, in H3K56Q mutants there was significantly higher recruitment of RNAPII in the promoter and ORF regions of the actively transcribing genes, while RNAPII recruitment was impaired in H3K56R mutants, compared to wild-type (Fig. 1C). To confirm that the observed effect of H3K56 acetylation on transcription of constitutively expressed genes, was a phenomenon that can be generalized for any housekeeping gene, the above results were confirmed for three more loci namely, *PMA1*, *ADH1* and *PHO84* (Fig. S1). Taken together, the above results indicated that acetylation of H3K56 was essential for recruitment of RNAPII during transcription of constitutively active genes.

H3K56 acetylation is dependent on H4K16 deacetylation. Previously we have reported that in constitutively active loci H4K16 deacetylation promotes H3K56

acetylation.[19] In order to elucidate the correlation between H4K16 deacetylation and H3K56 acetylation, we deleted Sas2, the major HAT responsible for H4K16 acetylation.[16,66,67] To validate the Δ*Sas2* mutant, we compared the levels of H4K16 acetylation in wild-type and the mutant and found that deletion of Sas2 reduced H4K16 acetylation levels significantly in constitutively active genes (Fig. S2). Thereafter we compared the levels of H3K56 acetylation in wild-type and Δ*Sas2* mutant. Our results indicated that in all the constitutively active genes tested, H3K56 acetylation was significantly higher in Δ*Sas2* mutant, compared to wild-type (Fig. 2A). The telomeric region (*TEL08R*) was used as the nontranscribing region control, to confirm that the observed H3K56 acetylation pattern was a signature related to transcribing loci. In trying to corroborate this with transcription, we observed that RNAPII occupancy was also

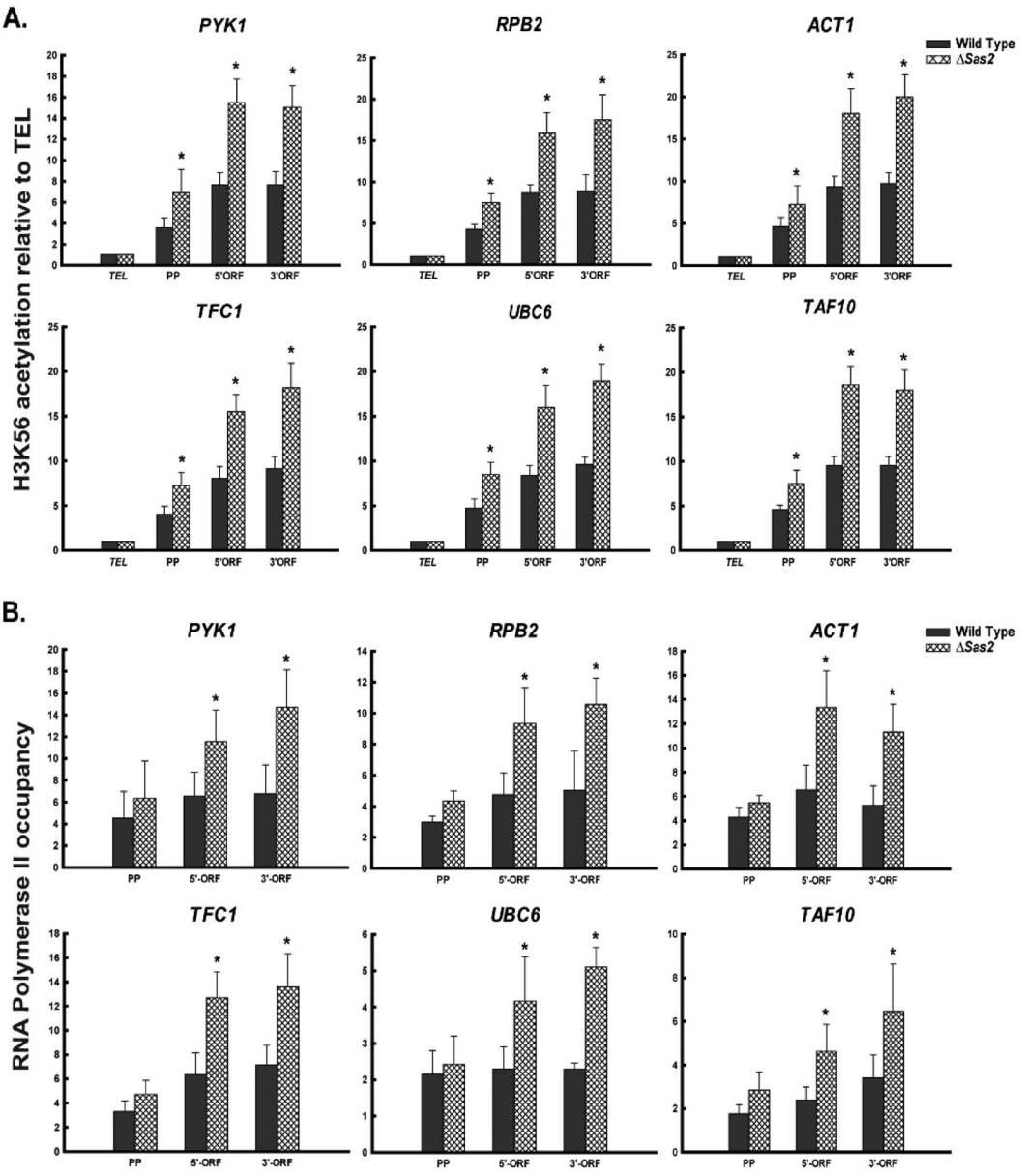

FIG 2 Comparative analyses of H3K56 acetylation levels and RNAP II occupancy in wild-type and Δ*Sas2* mutant. Chromatin of wild-type and Δ*sas2* mutant was immunoprecipitated with (A) anti-H3K56ac antibody (B) anti-RNAPII (8WG16) antibody and the occupancy was thereafter monitored by qPCR at the promoter (PP), 5′ORF and 3′ORF of *PYK1, RPB2, ACT1, TFC1, UBC6* and *TAF10* loci. H3K56 acetylation levels were normalized to ChIP data with anti-H3 antibody of the same regions and graphically represented relative to a nontranscribing control region of *TELO8R* denoted as *TEL*. RNAPII occupancy was normalized by input (1%) and graphically represented. Data represents the mean of three independent experiments with standard error of mean bars and * denote *t* test significant *P* values of <0.05.

significantly higher in Δ*Sas2* mutant, compared to wild-type (Fig. 2B). Notably, higher RNAPII recruitment was evident specifically in the ORF of the constitutively active genes that we tested. This was in consonance with previous reports that have indicated a negative correlation of Sas2 mediated H4K16 acetylation with RNAPII progression.[66] These results indicated that H4K16 deacetylation and the consequent higher H3K56 acetylation observed in the Δ*Sas2* mutant possibly aid RNAPII recruitment along the ORF of the constitutively expressed genes.

To understand the direct impact of H4K16 deacetylation on H3K56 acetylation, we deleted Hos2, the HDAC responsible for deacetylation of H4K16 in the euchromatin region.[68] To validate that Hos2 targets deacetylation of H4K16 residue, acetylation level of H4K16 was checked in WT and *Hos2*Δ mutants. Results indicated that in absence of Hos2, acetylation levels of H4K16 were significantly higher compared to wild-type, in all loci tested (Fig. S2). Cell survival assay with *Hos2*Δ mutants showed that under control conditions they have growth comparable to wild-type. To further correlate this with transcription, we did cell survival assay in presence of the transcription elongation inhibitor 6-azauracil (6-AU). In presence of 6-AU the *Hos2*Δ mutant showed increased sensitivity compared to wild-type. In addition, it was interesting to note that, H3K56Q mutation in *Hos2*Δ background exhibited increased resistance to 6-AU, compared to wild-type (Fig. 3A). This implied that H4K16 deacetylation along with H3K56 acetylation generated a chromatin landscape conducive for transcription progression in the gene body. Furthermore, H3K56 acetylation levels were reduced in *Hos2*Δ mutant, compared to wild-type, suggesting dependence of H3K56 acetylation on H4K16 deacetylation (Fig. 3B). H3K56 acetylation levels at the telomeric region (*TEL08R*) was used as the nontranscribing locus control. Furthermore, lack of H4K16 deacetylation impaired recruitment of RNAPII across the promoter and gene body of the constitutively expressed genes tested, indicating its requirement for progression of RNAPII in transcriptionally active loci (Fig. 3C). Taken together, the above results indicate that H3K56 acetylation is dependent on Hos2-mediated H4K16 deacetylation, and these two events are essential for active transcription.

In presence of constitutive acetylation of H3K56 the requirement for H4K16 deacetylation can be evaded. To further understand the crosstalk between H3K56 acetylation and H4K16 deacetylation, we generated the following sets of double mutants: H4K16RH3K56Q, H4K16QH3K56Q, H4K16RH3K56R, H4K16QH3K56R. As shown in Fig. 4A, cell survival assay under control conditions indicated that the double mutants H4K16RH3K56R and H4K16QH3K56R have moderately reduced growth compared to wild-type, the single mutants, and the double mutants H4K16RH3K56Q or H4K16QH3K56Q. Interestingly, cell survival assay in presence of the transcription inhibitor 6-AU showed that any single or double mutant that bore H3K56Q mutation exhibited increased resistance to 6-AU (Fig. 4A-right panel). This implied that acetylation of H3K56 is a critical factor that regulates efficient transcription. In consonance, we further observed that H3K56Q bearing double mutants showed significantly higher gene expression levels, compared to wild-type (Fig. 4B). Notably, such enhanced gene expression was observed in H3K56Q double mutants irrespective of whether H4K16 was mutated to R or Q along with. In contrast, double mutants bearing H3K56R mutation showed significantly reduced gene expression levels compared to wild-type, irrespective of H4K16 mutated to R or Q along with. Furthermore, RNAPII occupancy was higher in the double mutants bearing H3K56Q mutation compared to wild-type, irrespective of H4K16 acetylation status (Fig. 4C). These results not only indicate that H3K56 acetylation is an important event involved in transcription, but interestingly also imply that in presence of constitutive acetylation of H3K56 (H3K56Q) H4K16 deacetylation may be bypassed.

H4K16 deacetylation is required for Rtt109 recruitment in constitutively expressed genes. Rtt109 is the major HAT that is known to acetylate H3K56.[31,34,69,70] To understand how H4K16 deacetylation influences H3K56 acetylation, we expressed Myc-tagged Rtt109 in WT, H4K16R and H4K16Q cells. Thereafter ChIP with anti-Myc antibody was done to check for Rtt109 recruitment in the constitutively expressed genes in WT and the H4K16 mutant cells. We observed that in H4K16Q cells, which

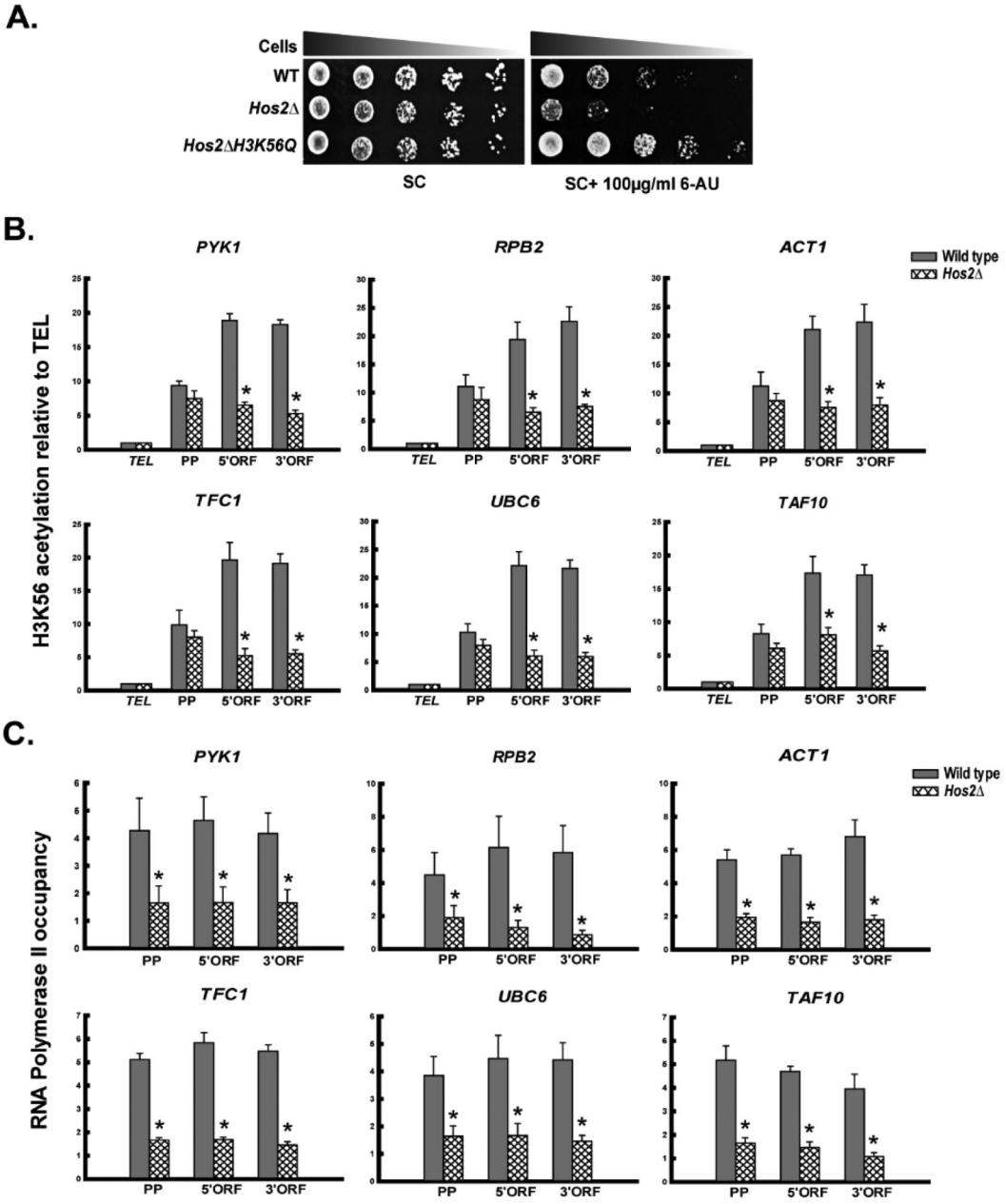

FIG 3 Hos2 facilitates H3K56 acetylation during transcription. (A) Ten-fold serial dilutions of wild-type, *Hos2*Δ and *Hos2*Δ*H3K56Q* were spotted on SC agar plates with or without 100 μg/mL 6-AU and growth sensitivity was monitored following an incubation period of 2–4 days. (B) Comparative analysis of H3K56 acetylation levels in wild type and *Hos2*Δ at the promoter (PP), 5′ORF and 3′ORF of *PYK1, RPB2, ACT1, TFC1, UBC6* and *TAF10* loci. H3K56 acetylation levels were normalized to ChIP data with anti-H3 antibody of the same regions and graphically represented relative to a nontranscribing control region of *TELO8R* denoted as *TEL*. (C) Chromatin of wild-type and *Hos2*Δ was immunoprecipitated with anti-RNAPII (8WG16) antibody and occupancy was checked at the promoter (PP), 5′ORF and 3′ORF of the loci mentioned above. The data normalized by 1% input was graphically plotted. Data represents the mean of three independent experiments with standard error of mean bars and * denote *t* test significant *P* values of <0.05.

lacked H4K16 deacetylation and mimicked a state of constitutive acetylation, Rtt109 recruitment was significantly impaired compared to wild-type, in the constitutively transcribing loci (Fig. 5). On the contrary, in presence of constitutive deacetylation of H4K16 (H4K16R), Rtt109 recruitment was favored compared to wild-type. Collectively these results suggest that H4K16 deacetylation promotes H3K56 acetylation by aiding recruitment of Rtt109 to the transcriptionally active loci.

Acetylation of H3K56 is dependent on active transcription. It was further necessary to understand whether acetylation of H3K56 was associated with active transcription. For this we checked the H3K56 acetylation levels in presence of another

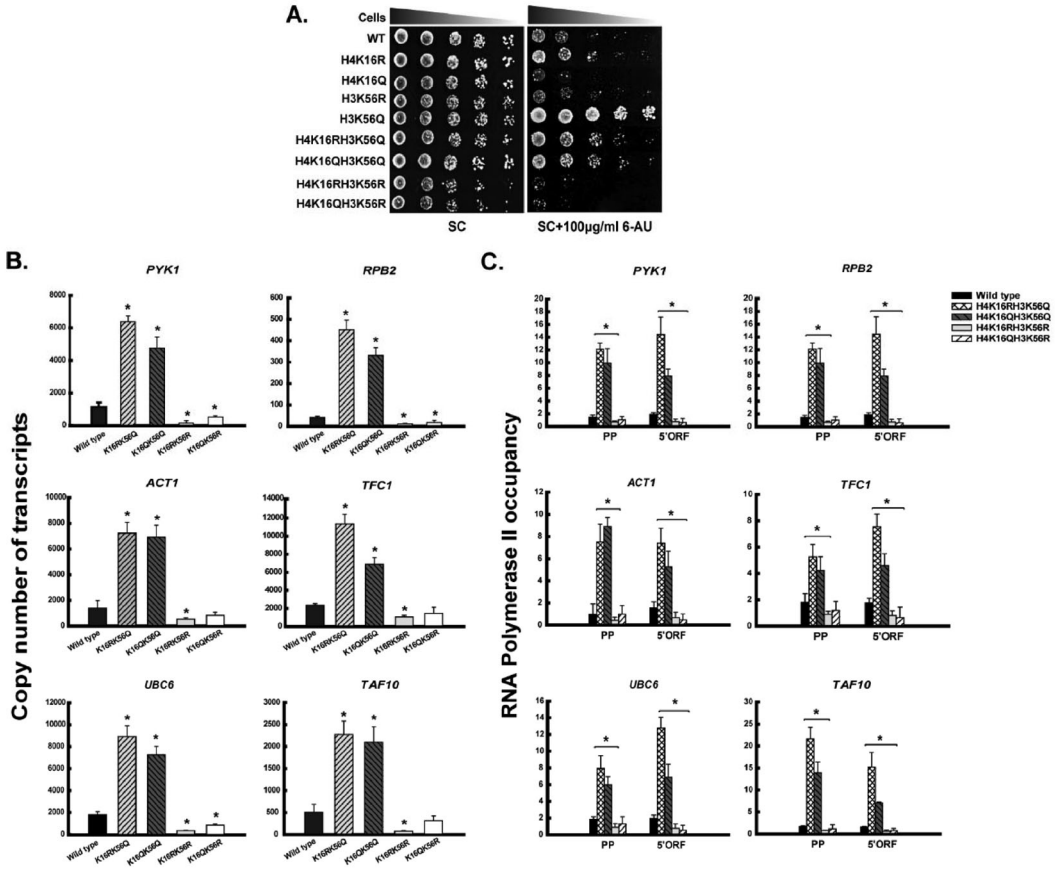

FIG 4 In H3K56Q mutant background H4K16 deacetylation can be bypassed. (A) Ten-fold serial dilutions of wild-type and mutants were spotted on SC agar plates with or without 100 µg/mL 6-AU and growth sensitivity was monitored following an incubation period of 2–4 days. (B) RNA was isolated from wild-type and mutants followed by RT-qPCR analyses using absolute quantification method as described in Materials and Methods. The copy number of transcripts of *PYK1, RPB2, ACT1, TFC1, UBC6* and *TAF10* in wild-type and mutants were graphically plotted. (C) ChIP analyses were done with anti-RNAPII (8WG16) antibody and occupancy was monitored at the promoter (PP) and 5′ORF of the loci mentioned above in wild-type and mutants. The data normalized by 1% input was graphically plotted. Data represents the mean of three independent experiments with standard error of mean bars and *denote *t* test significant *P* values of <0.05.

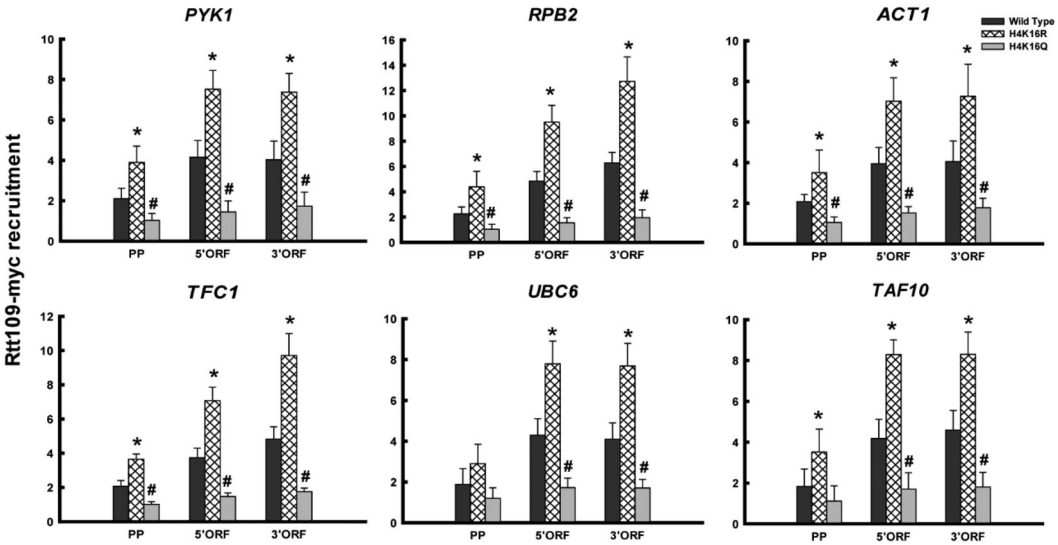

FIG 5 Comparative analysis of Rtt109 recruitment in wild-type and H4K16 mutants. Chromatin isolated from wild-type and H4K16 mutants was immunoprecipitated with anti-myc antibody to pull down myc-tagged Rtt109 and the occupancy was thereafter monitored by qPCR analyses at the promoter (PP), 5′ORF and 3′ORF of *PYK1, RPB2, ACT1, TFC1, UBC6* and *TAF10* loci. The data normalized by 1% input was graphically plotted. Data represents the mean of three independent experiments with standard error of mean bars. * and # denote *t* test significant *P* values of < 0.05.

transcription inhibitor 1,10-phenanthroline or PH. PH is a metal chelator that is known to significantly inhibit transcription in yeast.[71–75] Total cellular protein was isolated with or without PH treatment and immunoblotted with respective antibodies. To standardize PH treatment, we first performed immunoblot with antibody against H4K16 acetylation. Results indicated that, H4K16 acetylation levels increased upon inhibition of transcription, compared to when active transcription was present (Fig. 6A). This is in consonance with previous reports that have established negative

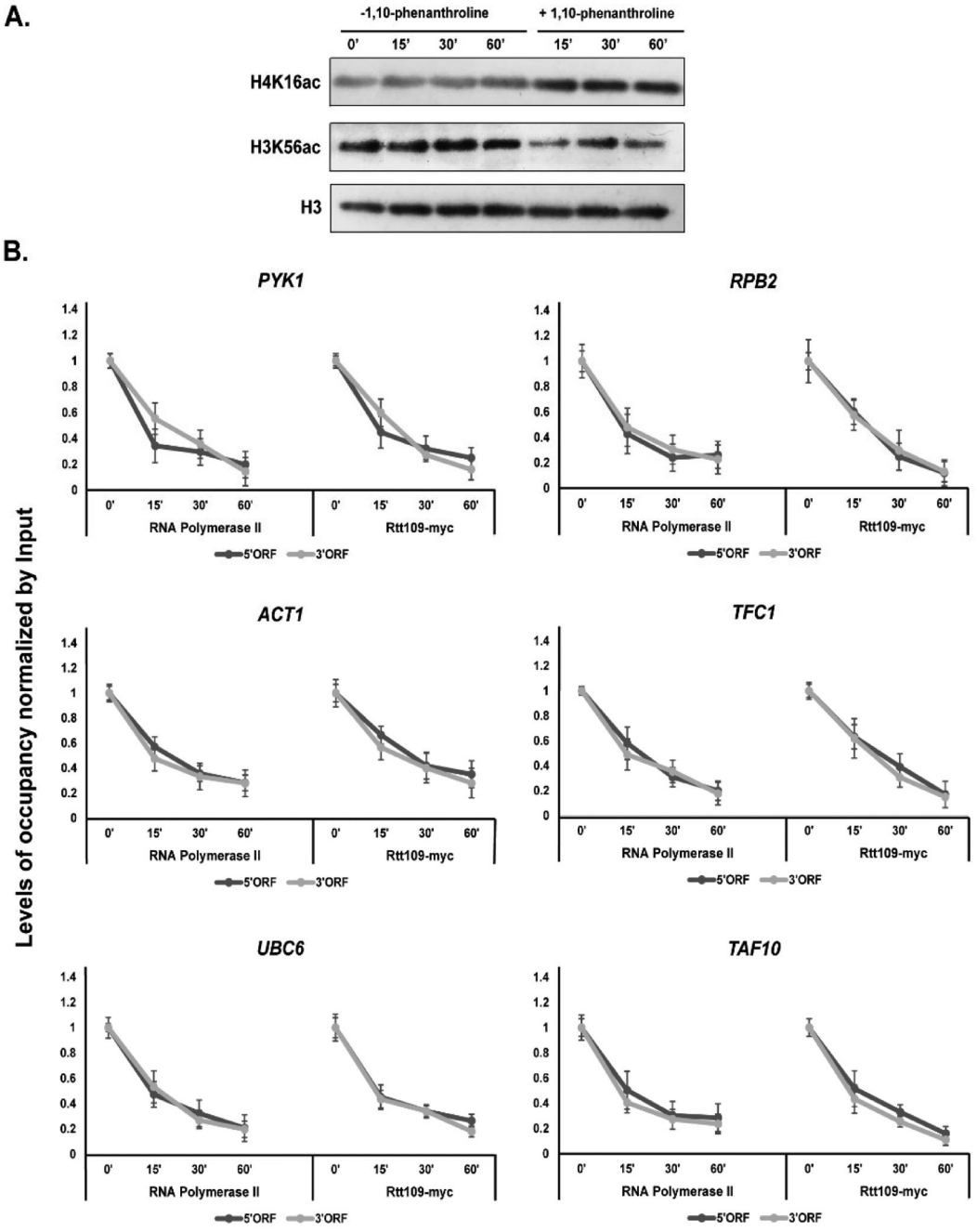

FIG 6 Transcription activity promotes H3K56 acetylation. (A) Equal amounts of whole cell extracts from wild-type cells treated with or without 1,10-phenanthroline for varying time periods as indicated were resolved on 16% SDS-PAGE and immunoblotted with anti-H4K16ac, anti-H3K56ac and anti-H3 antibody, respectively. (B) Chromatin isolated from wild-type cells treated with or without 1,10-phenathroline for 15, 30, and 60 min, respectively were immunoprecipitated with anti-RNAPII (8WG16) and anti-myc antibodies. RNAP II and Rtt109-myc occupancy of 1,10-phenathroline treated and untreated (0 min) sample was normalized by input (1%) and occupancy under conditions of transcription inhibition were graphically plotted relative to untreated (0 min), in the 5′ORF and 3′ORF of *PYK1, RPB2, ACT1, TFC1, UBC6,* and *TAF10* loci. Data represents the mean of three independent experiments with standard error of mean bars.

correlation between active transcription and H4K16 acetylation.[15,19,40,66,68,76,77] Immunoblot using antibody against H3K56 acetylation indicated that inhibition of transcription by PH treatment, distinctly reduced H3K56 acetylation, compared to untreated samples. This implied that unlike H4K16, acetylation of H3K56 is positively associated with active transcription and absence of transcription decreases H3K56 acetylation at the cellular level. To further establish the correlation between active transcription and H3K56 acetylation, we performed ChIP experiments with or without PH treatment. Simultaneous ChIP with antibody against RNAPII (8WG16) and myc tagged Rtt109 indicated that, PH treatment led to reduction in RNAP II recruitment along with reduction in Rtt109-myc occupancy. Such a reduction in RNAPII recruitment and Rtt109-myc occupancy was observed across the ORF of all tested constitutively active loci upon PH treatment, compared to untreated controls (Fig. 6B). These observations clearly indicate that the acetylation machinery of H3K56 is closely associated with active RNA Polymerase II and inhibition of transcription impairs recruitment of Rtt109 and subsequent acetylation of H3K56.

DISCUSSION

Understanding chromatin dynamics in the coding region of a transcribing gene is critical to understanding how RNAPII makes its way through the nucleosomes during transcription. To understand the "histone code" that regulates chromatin dynamism during transcription we had previously reported the effect of H4K16 deacetylation on expression of constitutively active genes. H4K16 acetylation is known to have an anti-correlation with transcription [15,19,40,58,66,68,76] and presence of constitutive deacetylation of H4K16 promote expression of constitutively active genes.[19] Our earlier observation indicated that deacetylation of H4K16 promotes acetylation of H3K56 residue. H3K56 is known to be a crucial histone residue whose acetylation has not only been correlated with nucleosome disassembly during transcription activation,[35,39,42] but also with transcription elongation.[34,41,45,47–49] Presently we observed that H3K56 acetylation is significantly higher in the coding regions of constitutively expressed genes compared to their promoter. As evident, such hyperacetylation positively correlated with transcription (Fig. 1). Recent studies have reported that unwrapping of the DNA at the entry/exit site mediated by H3K56 acetylation enables RNAPII to translocate through nucleosomes.[48] In consonance, we observed that lack of H3K56 acetylation (H3K56R) significantly impeded recruitment of RNAPII, especially in the ORF of active genes. Collectively, our results indicated that H3K56 acetylation in the coding region is an important event during transcription of constitutively expressed genes. Strikingly, H3K56 acetylation pattern in constitutive genes (Fig. 1) is in negative correlation with the pattern of H4K16 acetylation which is significantly low in the coding region, compared to promoter.[19] We further observed that, deacetylated state of H4K16 in Δ*Sas2* mutant promotes acetylation of H3K56 in the coding region of constitutively active loci, suggesting the essentiality of H4K16 deacetylation for H3K56 acetylation. This, coupled with the presence of significantly higher levels of RNAPII in the coding regions of Δ*Sas2* mutants, compared to wild-type, endorsed the fact that H4K16 deacetylation and effectively H3K56 acetylation influences RNAPII progression within the gene bodies (Fig. 2). In actively transcribing loci, H4K16 deacetylation is primarily catalyzed by the HDAC Hos2 for regulating gene activation.[68] The observation that in presence of transcription elongation inhibitor 6-AU, *Hos2*Δ mutants show increased growth sensitivity compared to wild-type, indicated a role of Hos2-mediated H4K16 deacetylation during transcription. Furthermore, in absence of Hos2, H3K56 acetylation and RNAPII recruitment along the active gene loci reduced significantly (Fig. 3). It therefore implied that Hos2 mediated H4K16 deacetylation is important for progression of RNAPII during transcription. Notably, constitutive acetylation of H3K56 in absence of Hos2 (*Hos2*Δ*H3K56Q*) could restore the growth sensitivity to 6-AU exhibited by *Hos2*Δ mutant. This implied that Hos2-mediated deacetylation of H4K16 is essential for H3K56 acetylation and that in presence of constitutive acetylation of H3K56, Hos2 function

can be bypassed. In consonance with our findings, it has been recently reported in *Beauveria bassiana* that Hos2 deletion leads to reduced acetylation of H3K56.[78] Collectively, our findings make it explicit that Hos2 mediated deacetylation of H4K16 is a pre-requisite for H3K56 acetylation, and these events are required for RNAPII mediated effective transcription.

Our observations on growth sensitivity to 6-AU clearly indicated that while constitutive acetylation of H4K16 (H4K16Q) has anticorrelation with transcription, constitutive acetylation of H3K56 (H3K56Q) promotes transcription. What is to be further noted is that presence of constitutive acetylation of H3K56 conferred resistance to 6-AU irrespective of the H4K16 acetylation status (Fig. 4). Subsequent, gene expression profiles and RNAPII occupancy with the double mutants further implied that H3K56 acetylation was vital for transcription and more importantly in presence of constitutive H3K56 acetylation requirement for H4K16 deacetylation could be evaded. Furthermore, the observation that presence of constitutive H3K56 acetylation (H3K56Q) made transcription independent of H4K16 acetylation status was significant. It implied that, H4K16 deacetylation can promote transcription only in presence of effective H3K56 acetylation. Thus, the transcription defect exhibited by H4K16Q could be restored in presence of constitutive acetylation of H3K56 (H4K16QH3K56Q). This further emphasizes that H3K56 acetylation is critical for transcription of constitutively active genes and that deacetylation of H4K16 is primarily required to promote acetylation of H3K56 during the process. Indeed, recruitment of Rtt109, the HAT for H3K56ac, was found to be dependent on H4K16 acetylation status. Presence of H4K16 deacetylation (H4K16R) promoted Rtt109 recruitment whereas constitutive acetylation of H4K16 (H4K16Q) significantly impeded Rtt109 recruitment in active genes (Fig. 5). This implied that H4K16 deacetylation must precede Rtt109 recruitment and the resultant H3K56 acetylation. In transcribing loci, several bromodomain containing HATs such as Gcn5[79,80] are known to bind to H4K16 acetylated histones. It is possible that such binding hinders the association of H3K56 acetylation machinery with its target residue. H4K16 deacetylation may thus be a probable mechanism by which the respective HAT Rtt109, gets effectively recruited for H3K56 acetylation. Earlier it has been reported that absence of H3/H4 N-terminal tails compromises Rtt109 mediated acetylation of H3K56, indicating that H3/H4 tails have a regulatory role in H3K56 acetylation.[33,36] Further investigations are required in this direction to understand how exactly H4K16 deacetylation promotes H3K56 acetylation.

It is known that Hos2 mediated H4K16 deacetylation is targeted to active genes by the RNAPII Ser 5 CTD.[58,59,81] Active transcription is therefore required for H4K16 deacetylation. In consonance we observed that inhibition of transcription with 1,10 phenanthroline[71,72] causes H4K16 hyperacetylation at the cellular level further indicating that deacetylation of H4K16 is transcription dependent (Fig. 6). In trying to understand whether the H4K16 deacetylation mediated H3K56 acetylation event was also dependent on active transcription, we observed that H3K56 acetylation was conspicuously reduced upon transcription inhibition at the cellular level. H3K56 residue is present on the lateral surface of the histone octamer which is embedded in nucleosomal DNA and thus far has been reported to be acetylated primarily on free histones.[33] However, in consonance with Schneider et al.[34] we observed that Rtt109 promotes H3K56 acetylation within the nucleosome dense coding regions of actively transcribing genes, raising a possibility that Rtt109 may acetylate nucleosomal H3, as well. Moreover, upon transcription inhibition, Rtt109 recruitment within coding regions of constitutive genes decreased in a manner that strongly correlated with RNAPII dissociation (Fig. 6). This implied that Rtt109 function was closely associated with the active form of RNAPII. However, we could not observe any direct physical interaction between RNAPII and Rtt109 either due to lack of any such interaction or maybe such interaction could not be captured under our experimental conditions. Interestingly, recent studies have suggested that the localized chromatin disruptions caused by RNAPII promotes recruitment of HATs to gene bodies.[75] In fact, in mammals it has been shown that, disruption

of nucleosomes by nucleosome-destabilization factor (NDF) allows p300-CBP mediated H3K56 acetylation even on nucleosomal histones.[82] A similar mechanism might be involved in recruitment of Rtt109 and consequent acetylation of H3K56 during transcription in a chromatinized template. Future investigations in this regard would help to shed more light on the underlying mechanisms. However, the strong correlation between Rtt109 and RNAPII occupancy on chromatin clearly suggest that transcriptional activity in the coding region promotes Rtt109 recruitment and effectively H3K56 acetylation in a manner that is essentially dependent on H4K16 deacetylation.

In summary, our findings reveal that H3K56 acetylation is crucial for RNAPII progression in the gene body of actively transcribing genes. Hos2-mediated H4K16 deacetylation is dependent on active transcription and essentially directs H3K56 acetylation. This becomes evident as constitutive presence of H3K56 acetylation can alleviate the need for Hos2-mediated H4K16 deacetylation during transcription. H4K16 deacetylation along with H3K56 acetylation together generate a "histone code" that facilitates RNAPII passage through the gene body and forms a crucial combination of histone modifications required for successful transcription in active loci.

MATERIALS AND METHODS

Yeast strains. The procedure for generation of yeast strains has been previously discussed in detail.[18,19,83] Briefly, both copies of genomic histone H3-H4 were deleted from *S. cerevisiae* strain WY121 bearing plasmid pJL001 (*CEN URA3 HHT2-HHF2*) as the only source of histone H3-H4 and *URA3* as the counter-selectable marker. Plasmid pEMH7 (*CEN TRP1 HHT2-HHF2*) containing one copy of H3-H4 with counter-selectable marker *TRP1* was transformed into WY121 followed by growth in presence of 5-FoA (5′- Fluoroorotic acid) consequently leading to the loss of plasmid pJL001 and the subsequent generation of wild type strain. The plasmids pEMH33, pEMH35 bearing H4K16R and H41K6Q point mutations in *HHF2* gene and plasmids pEMH129, pEMH108 bearing H3K56R and H3K56Q point mutations in *HHT2* gene, respectively, were isolated from *Escherichia coli* and transformed similarly into WY121 yeast strain followed by plasmid shuffling to generate histone H4 and H3 mutant strains H4K16R, H4K16Q, H3K56R and H3K56Q, respectively. The transformed plasmids were isolated from yeast cells and sequenced to confirm the presence of the desired mutations.

For the generation of double mutants, plasmids pEMH33 and pEMH35 bearing H4K16R and H41K6Q point mutations in *HHF2* gene were subjected to site-directed mutagenesis[84] to generate point mutations H3K56R and H3K56Q in the *HHT2* gene of the respective plasmids. After sequencing confirmations, the plasmids bearing point mutations H4K16RH3K56Q, H4K16QH3K56Q, H4K16RH3K56R and H4K16QH3K56R, respectively were transformed into WY121 followed by plasmid shuffling in presence of 5-FoA to generate the respective double mutant strains. WY121 strain and pEMH plasmids were gifts from Dr John Wyrick (Washington State University, USA) and Prof. J.D. Boeke (Johns Hopkins University School of Medicine, USA), respectively. Δ*Sas2* mutant was generated as previously discussed.[19] For this study, *Hos2*Δ and *Hos2*Δ*H3K56Q* strains were generated similarly by the PCR-based homologous recombination-mediated gene deletion method.[85] Plasmid pFA6-KANMX4 was a gift from Prof. Shubho Chaudhuri (Bose Institute, Kolkata, India).

For C-terminal myc-tagging of *RTT109* in wild type, H4K16R and H4K16Q strains, PCR based homologous recombination method was employed.[85] A linear DNA cassette containing the KANMX6 module and flanking regions homologous to regions immediately upstream and downstream of the stop codon of *RTT109* gene was amplified from shuttle vector, pFA6A-13myc-KANMX6 and used to transform wild-type, H4K16R and H4K16Q strains, respectively and allowed to grow on G418 for selection of positive transformants. The tagging of wild-type and H4K16 mutants was confirmed by Western blot analysis. Plasmid pFA6A-13myc- KANMX6 was procured from Addgene (Addgene plasmid # 39294; http://n2t.net/addgene:39294; RRID: Addgene_39294)

Gene expression analysis. Yeast cells grown till mid-log phase with an $O.D_{600}$ of ∼0.7 (∼1 × 107 cells/mL) were collected to isolate RNA.50 mL cell pellets were flash-frozen in liquid N_2. To the frozen cell pellet TRIzol® reagent was added and allowed to defrost on ice. Cell pellets were resuspended in TRIzol® reagent and transferred to 1.5 mL tubes containing DEPC treated acid-washed glass beads. The cells were disrupted by vortexing with 1 min rest in between cycles. Lysed cells were centrifuged at full speed for 15 min at 4 °C to pellet debris. The supernatant was subjected to chloroform extraction and subsequent precipitation of RNA pellets with isopropanol. Pellets were washed with 75% ethanol, air-dried, and dissolved in RNAse free water. 20 μg of RNA was subjected to DNase I treatment followed by TRIzol®: chloroform extraction and isopropanol precipitation. Five micrograms of total RNA was used for reverse transcription using Revertaid RT (Thermo Scientific) as per manufacturer's instructions. An absolute quantification method was employed to obtain the copy number of transcripts for every gene in wild-type and mutants which is equivalent to the mRNA copy numbers.[19,86] For this, as previously described[19], a standard curve was prepared from serial dilutions of the cDNA ranging from 1/101 to 1/109 to serve as template for qPCR with primers for the six constitutive genes and the C_T values were used to plot a standard curve depicting C_T values on the Y-axis for the corresponding number of amplicons (ng) on the X-axis for each primer set. Subsequently, qPCR was performed with undiluted cDNA

samples and the C_T values of the expressed genes were plotted on the standard curves to calculate the actual number of transcripts expressed for each gene using the standard formula: (amount of dsDNA in ng * Avogadro No.)/(Base pair size of dsDNA) *(330*2*D. F). The dilution factor (DF) was 109 and the amount of dsDNA present in ng was estimated as values of "x" from the standard curve plot: $y = m.\ln(x) + c$ considering the C_T values of the expressed genes as "y." Triplicate qPCRs were performed twice for each biological replicate ($n = 3$) of every strain. To denote statistical significance of the difference in gene expression levels between wild-type and mutant strains a two-tailed independent Student's *t* test was performed and the results with *P* value of < 0.05 were significant.

6-AU sensitivity assay. Ten-fold serial dilutions of exponentially grown yeast cells OD_{600} of 0.6 - 0.7 ($\sim 0.9 \times 10^7$ cells/mL) in yeast extract-peptone-dextrose (YPD) were spotted on SC medium with or without 100 μg/mL 6-AU (Sigma-A1757) and incubated at 30 °C. The control plates without 6-AU were incubated at 30 °C for 2 days and the 100 μg/mL 6-AU treated plates were incubated for 4 days at 30 °C.

Phenanthroline treatment. For phenanthroline treatment, mid-log phase yeast cells with an OD_{600} of ~0.7 (~1 × 107 cells/mL) were used, an untreated sample (0 min) was separated, the rest of the cells were divided into two equal halves, one-half of the cells were treated with 200 μg/mL 1,10-phenanthroline (Sigma- P9375) dissolved in 100% ethanol and the other half of the cells were simultaneously treated with 100% ethanol without 1,10-phenanthroline. The samples that were only subjected to 100% ethanol were simultaneously incubated with phenanthroline treated samples at 30 °C for a period of 15, 30, and 60 min, respectively. The phenanthroline treated and untreated cells were used for Western blot analysis and chromatin immunoprecipitation assay.

Western blot analysis. Whole cell extracts were prepared as per the protocol previously described.[19] Protein concentration was measured by Bradford assay and equal amounts of protein (10 μg) from each sample were electrophoresed on a 16% SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5% NFD in 1X TBS and incubated overnight at 4 °C with the following primary antibodies: antihistone H4 Acetyl K16 (Cell Signaling Technology – E2B8W), antihistone H3 acetyl K56 (Abcam – ab195478), and antihistone H3 (Biobharati, India, BB-AB0055). Following an incubation with appropriate horseradish peroxidase (HRP) – conjugated secondary antibody, membrane was incubated with a chemiluminescent substrate (SuperSignal® West Pico Chemiluminescent Substrate by Thermo Scientific) and visualized on an autoradiographic film.

Chromatin immunoprecipitation. The detailed protocol of chromatin immunoprecipitation assay has been previously described.[18,19,83] Briefly, cells were collected in their mid-log phase OD_{600} of ~0.7 (~1 × 107 cells/mL) and formaldehyde cross-linked to capture protein-DNA complexes. The antibodies used for ChIP assay were as follows: antihistone H3 acetyl K56 (Abcam – ab195478), anti-RNA polymerase II [8WG16] (Abcam – ab817), anti-myc tag [9E10] (Abcam- ab32) and antihistone H3 (Biobharati, India, BB-AB0055). For each ChIP, qPCR analysis was done as previously described.[18,19,83] Histone modification levels were calculated by the formula $2^{(C_T(\text{Input}) - C_T(\text{IP}))}$ and normalized by the levels of histone H3. H3K56ac levels were graphically represented as relative to a nontranscribing control region of *TELO8R*. RNA polymerase II and Rtt109-myc occupancy were calculated as a percentage of input (1%) after extrapolating the input to 100%. The assay was performed in triplicates for every biological replicate ($n = 3$). Statistical significance was calculated using a two-tailed independent Student's *t* test and the results with *P* value of < 0.05 were significant.

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AUTHOR'S CONTRIBUTION

RNC and PK designed and conceptualized the experiments. PK and PS performed the experiments. PK and RNC analyzed the data and wrote the manuscript. The manuscript has been read and approved by all authors.

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DATA AVAILABILITY STATEMENT

All strains, plasmids and reagents used in this study are available upon request. The authors confirm that all data supporting the findings of this study are available within

the main article or in the supplementary material and can also be found at figshare doi:10.6084/m9.figshare.23931501.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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